DEVELOPMENTAL DYNAMICS 212:364–372 (1998)

Expression Pattern of Two *Frizzled*-Related Genes, *Frzb-1* and *Sfrp-1*, During Mouse Embryogenesis Suggests a Role for Modulating Action of *Wnt* Family Members

BANG H. HOANG,[1] J. TERRIG THOMAS,[2] FADI W. ABDUL-KARIM,[3] KRISTEN M. CORREIA,[4] RONALD A. CONLON,[4] FRANK P. LUYTEN,[2] AND R. TRACY BALLOCK[1]*

[1]*Department of Orthopaedics, University Hospitals of Cleveland, Rainbow Babies and Childrens Hospital, and Case Western Reserve University, Cleveland, Ohio*

[2]*Craniofacial and Skeletal Diseases Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland*

[3]*Department of Pathology, University Hospitals of Cleveland, Cleveland, Ohio*

[4]*Department of Genetics, Case Western Reserve University, Cleveland, Ohio*

ABSTRACT **Wnt proteins have been implicated in regulating growth and pattern formation in a variety of tissues during embryonic development. We previously identified *Frzb-1*, a gene which encodes a secreted protein with homology in the ligand binding domain to the Wnt receptor Frizzled, but lacking the domain encoding the putative seven transmembrane segments. Frzb-1 has recently been shown to bind to Wnt proteins in vitro, and to inhibit the activity of *Xenopus* Wnt-8 in vivo. We report now that *mFrzb-1* and *Wnt* transcripts display both complementary and overlapping expression patterns at multiple sites throughout embryonic development. By Northern analysis, the expression of *mFrzb-1* in the developing mouse embryo is greatest from 10.5 to 12.5 days postcoitum (dpc). In the early embryo, *mFrzb-1* is expressed in the primitive streak, presomitic mesoderm, somites, and brain. Later, *mFrzb-1* exhibits sharp boundaries of expression in the limb bud, branchial arches, facial mesenchyme, and in cartilaginous elements of the appendicular skeleton. We conclude from these experiments that *Frzb-1* is expressed at a time and location to modulate the action of Wnt family members during development of the limbs and central nervous system. *Dev. Dyn. 1998;212:364–372.***

Key words: mouse; embryogenesis; frizzled-like proteins; Wnt; limb

INTRODUCTION

The *Wnt* gene family represents a large and diverse group of signaling molecules involved in the proliferation and differentiation of a variety of cell types (Nusse and Varmus, 1992). Localization and gene targeting approaches have recently uncovered particularly important roles of *Wnt* family members not only in the development of the nervous system, but also in regulating pattern formation of the limbs (McMahon and Bradley, 1990; Parr and McMahon, 1995). The dorsal-ventral (D-V) axis of the limb is established in part by a Wnt-7a signal from the dorsal ectoderm and involves the induction of the homebox gene *Lmx-1* in the dorsal mesenchyme (Riddle et al., 1995). Targeted disruption of the *Wnt-7a* in mice results in transformations of dorsal limb structures to ventral fates (Parr and McMahon, 1995).

The *Drosophila* gene *Frizzled* is a member of a gene family which regulates tissue polarity, and when mutated, produces disorganization of cuticular structures of the wings. The *Frizzled* gene product is a cell surface protein with an N-terminal cysteine-rich domain (CRD) and seven putative transmembrane segments. Members of the frizzled family can signal in response to Wnt binding to the CRD domain (Bhanot et. al., 1996; Yang-Snyder, et al., 1996; He et al., 1997; Perrimon, 1996).

We recently reported the discovery of Frzb-1 (Hoang et al., 1996), a secreted protein isolated from bovine articular cartilage which also contains an N-terminal CRD with homology to all Frizzled-class proteins, but lacks the putative seven- transmembrane domain. Frzb-1 is also capable of binding to members of the Wnt family in vitro, presumably through its N-terminal CRD (Wang et al., 1997; Leyns et al., 1997). In addition to its ability to bind to Wnt-8 and Wnt-1, Frzb-1 also acts as a functional inhibitor of Wnt-8 activity in Xenopus embryos (Leyns et al., 1997; Wang et al., 1997). Recently, several other molecules have been described, including SDF5 (Shirozu et al., 1996) Fritz (Mayr et al., 1997), hFRP (Finch et al., 1997), Sfrp-1, Sfrp-2, and Sfrp-4 (Rattner et al., 1997,) Crescent (Pfeffer et al., 1997), SARP1, SARP2, and SARP3 (Melkonyan et al., 1997), and FrzA (unpublished data), which are homologous to Frzb-1 in the CRD domain and also lack the putative transmembrane domain. Com-

Grant sponsor: University Hospitals of Cleveland; Grant sponsor: Rainbow Babies and Childrens Hospital.

*Correspondence to: R. Tracy Ballock, Department of Orthopaedics, University Hospitals of Cleveland, Rainbow Babies and Childrens Hospital, and Case Western Reserve University, Cleveland, OH 44106.

Received 3 December 1997; Accepted 18 February 1998

parative analysis of these proteins reveals that SDF5 is identical to SARP1, and Sfrp-2; Sfrp-1 is identical to hFRP, FrzA and SARP2; Frzb-1 is identical to Sfrp-3 and Fritz; and Sfrp-4, SARP3 and Crescent appear to be unique members of this family to date.

For Frzb-1 to exert a Wnt-modulating role, it must be present at the correct time and at the correct location during embryonic development. In the present study, we describe the spatiotemporal pattern of *mFrzb-1* expression during mouse embryonic development and compare this to the expression of the related genes *Sfrp-1*, *Wnt-7a*, *Wnt-7b*, and *Wnt-5a* in the developing limbs and central nervous system.

RESULTS

Cloning and Identification of Murine Frzb-1

Extensive restriction enzyme mapping and sequence analysis of the genomic clone mFrzb 3.1 revealed the presence of an exon encoding the 5′ untranslated region (UTR) and the entire CRD of murine *Frzb-1*. Comparison of the genomic sequence to the previously published bovine and human *Frzb* cDNAs (Hoang et al., 1996) confirmed it to be exon 1 of murine *Frzb-1*. This exon encodes approximately 300 bp of the 5′-UTR followed by an open reading frame (ORF) of 159 amino acids. The 3′-end of the exon is defined by the presence of a donor splice junction. During the course of our present investigation, three independent groups have also isolated murine *Frzb-1* cDNAs, referred to as *Frzb-1* (Leyns et al., 1997), *Sfrp-3* (Rattner et al., 1997), and *mFiz* (Mayr et al., 1997).

Expression of mFrzb-1 by Northern Analysis

To begin to characterize the temporal expression pattern of *mFrzb-1* during embryogenesis, we performed Northern analysis using total cellular RNA from 9.5-15.5 dpc whole mouse embryos, embryonic mouse limb buds, and neonatal rat organs. In mice, *mFrzb-1* appears to be expressed as a single RNA species of approximately 2.0 kb throughout development from 9.5 to 15.5 dpc, (Fig. 1A). To determine if this *mFrzb-1* expression was linked to limb development, Northern analysis was performed on RNA extracted from microdissected mouse limbs at two different stages, 12.5 and 15.5 dpc, and compared to RNA extracted from the whole embryo. At 12.5 dpc, *mFrzb-1* transcripts are clearly enriched in the limb RNA fraction (Fig. 1B).

In neonatal rat organs, the highest levels of *Frzb-1* mRNA are found in the epiphyseal cartilage. Rat *Frzb-1* transcripts are also abundant in neonatal kidney, lung, and spleen, whereas expression is weak but detectable in neonatal heart and brain (Fig. 1D).

A previous analysis of the bovine and human cDNAs (Hoang et al., 1996) suggests that the heterogeneity in transcript size between species is a result of alternative polyadenylation sites in the 3'-UTR of the mRNA. For example, the bovine cDNA clone has two distinct polyadenylation sites that can be used to produce transcripts of 1.7 and 2.4 kb in length (Hoang et al., 1996).

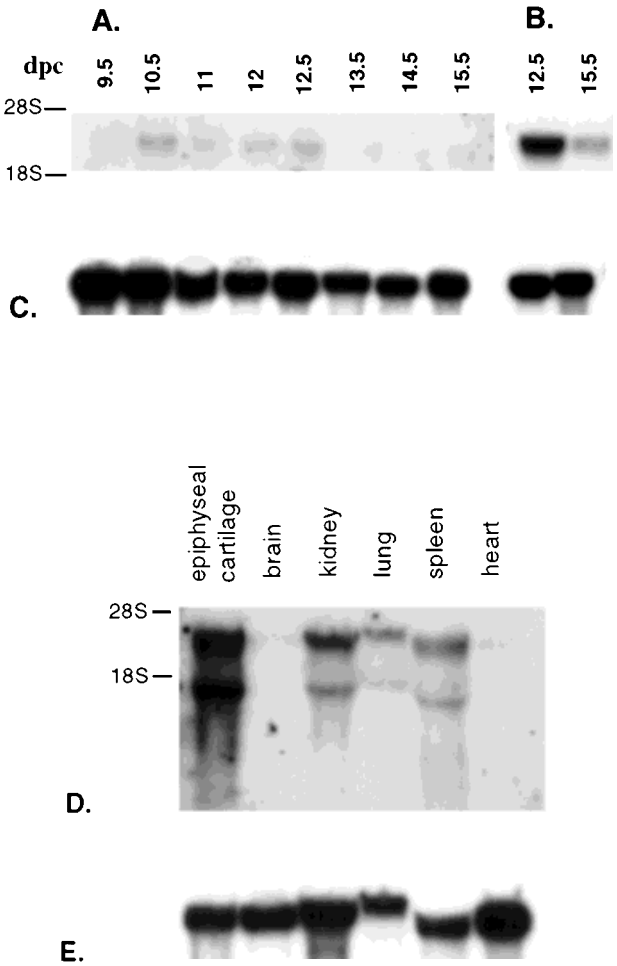

Fig. 1. Northern blot of *Frzb-1* gene expression in staged mouse embryos and in neonatal rats. Total RNA (20 μg) from whole mouse embryos (**A**), mouse fetal limbs (**B**), and indicated organs from neonatal rats (**D**) were analyzed using a α-^{32}P-labelled *mFrzb-1* probe (described in Experimental Procedures). The membranes were then stripped and probed with rat glyceraldehyde-6-phosphate dehydrogenase (GAPDH) (**C**, **E**) to normalize for loading as well as the quality of the RNA. Positions of the 18S and 28S rRNA bands are indicated. The membranes were exposed to radiographic films for 10 days (A), 3 days (B), 5 days (D), and 12 hr (C, E).

The rat *Frzb-1* mRNA also appears to display two transcripts of approximately 2.5 kb and 1.5 kb in size (Fig. 1D).

Expression of mFrzb-1 Between 7.5 and 9.5 dpc

Staged mouse embryos from 7.5 to 10.5 dpc were used to study expression of *mFrzb-1* by wholemount in situ hybridization. Because the wholemount technique is not suitable for examining embryos at later stages, expression of *mFrzb-1* at stages later than 11.5 dpc was studied by in situ hybridization performed on tissue sections. Concurrent control experiments using a sense probe did not produce specific signals at any developmental stage.

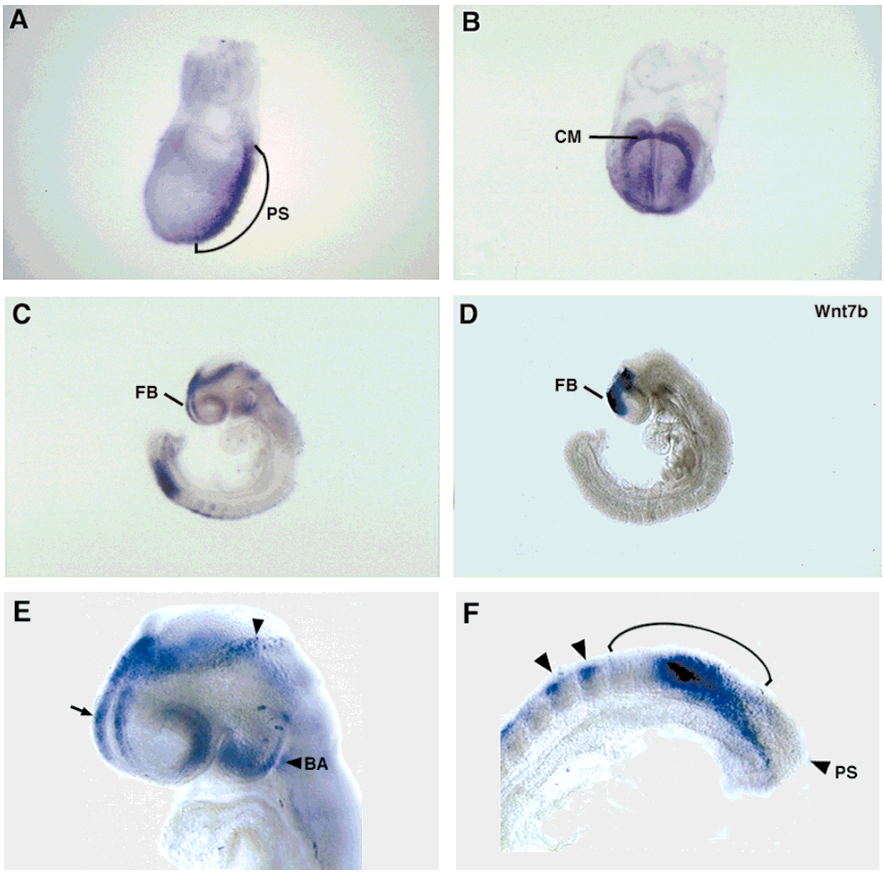

Fig. 2. Localization of *mFrzb-1* and *Wnt-7b* transcripts in early development. All samples were stained by wholemount in situ hybridization. **A,B:** Detection of *mFrzb-1* mRNA in 7.5 dpc mouse embryos. Expression of *mFrzb-1* (**C**) and *Wnt-7b* (**D**) at 8.75 dpc. **E:** Expression of *mFrzb-1* at 8.5 dpc in the forebrain (arrow), midbrain (arrowhead) and branchial arch. **F:** A lateral view of an 8.75 dpc embryo showing the expression of *mFrzb*-1 in the presomitic mesoderm (bracket) and in somites (arrowheads). BA, branchial arch; CM, cardiac mesoderm; FB, forebrain; PS, primitive streak.

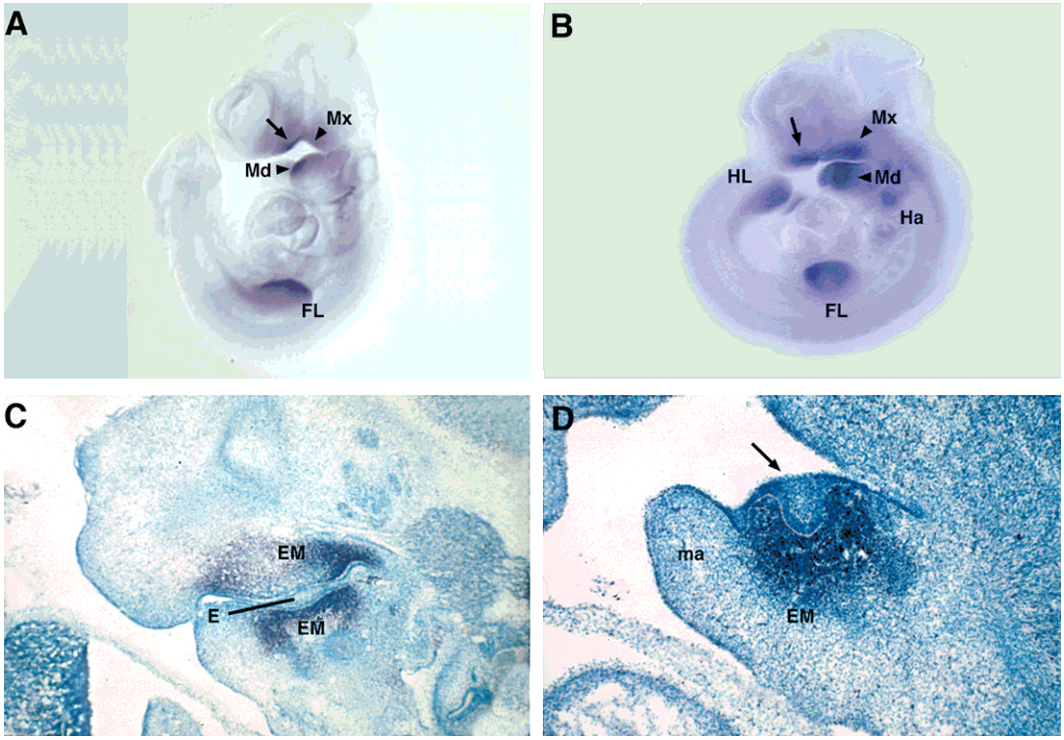

Fig. 3. Craniofacial expression of *mFrzb-1*, examined by in situ hybridization. Detection of *mFrzb-1* mRNA at 9.5 dpc (**A**) and 10.5 (**B**) by wholemount in situ techniques. Arrows point to the ventral facial mesenchyme expression. **C,D:** Detection of *mFrzb-1* at 12.5 dpc by tissue section in situ hybridization. Arrow indicates the thickened oral epithelium. E, oral epithelium; EM, ectomesenchyme; FL, forelimb; Ha, hyoid arch; HL, hindlimb; ma, mandible; Md, mandibular division of the first branchial arch; Mx, maxillary division of the first branchial arch.

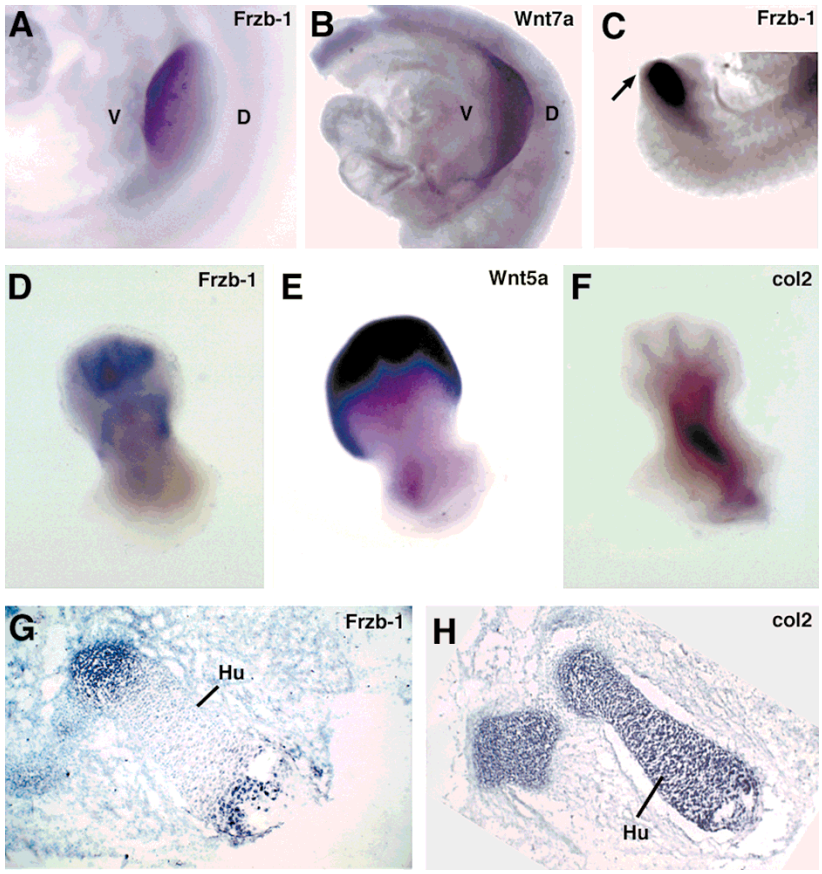

Fig. 4. Expression profile of *mFrzb-1*, *Wnt*s, and type II collagen during limb development, revealed by wholemount (A-E) and by tissue section in situ hybridization (F and G). A-C: Distribution of *Frzb-1* (**A**) and *Wnt-7a* (**B**) transcripts in the forelimb at 9.5 dpc, and *Frzb-1* (**C**) at 10.5 dpc in the hindlimb (arrow). Expression of *Frzb-1* is initially ventral-specific, as seen in the forelimb at day 9.5, then extends more dorsally, as seen in the hindlimb at day 10.5. D-F: Forelimb buds at 11.5 dpc, hybridized with *Frzb-1* (**D**), *Wnt-5a* (**E**), and collagen II (**F**) probes. The expression of *Frzb-1* is no longer detected in the ventral portion of the limb by day 11.5 and is found primarily in the perichondrial region surrounding the condensing mesenchyme. G,H: In situ hybridization of tissue sections through the cartilaginous anlage of the forelimb at 13.5 dpc showing the distribution of *Frzb-1* (**G**) and collagen II (**H**) transcripts. *Frzb-1* expression is no longer occurring in the perichondrium, but is found in the chondroblasts at the proximal and distal ends of the condensing cartilaginous anlage. D, dorsal; V, ventral; Hu, humerus.

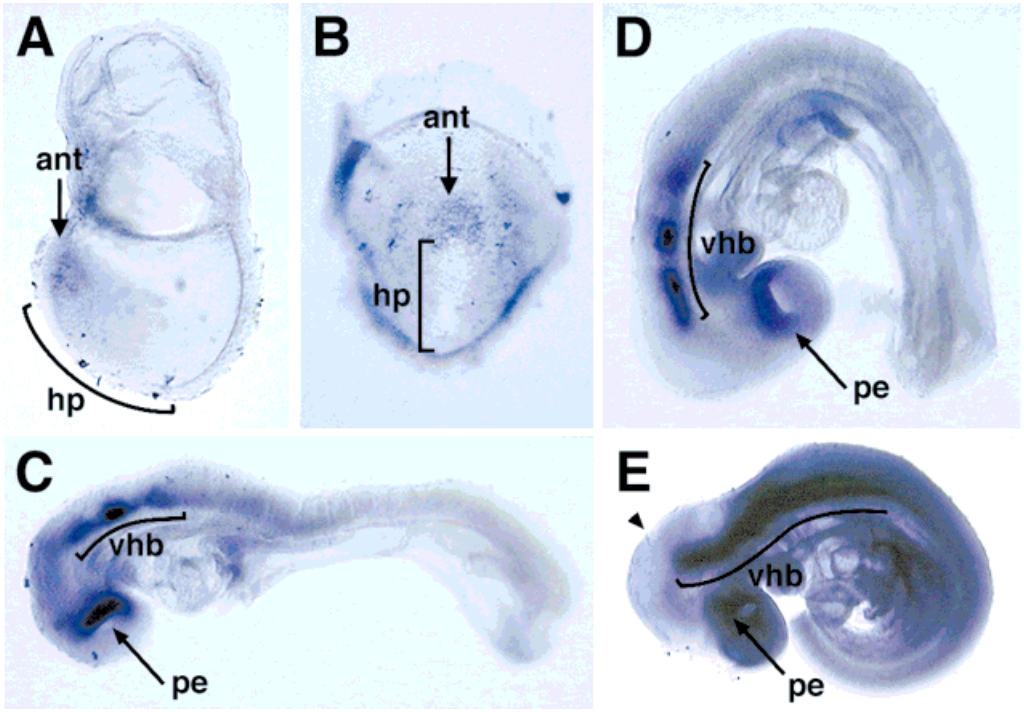

Fig. 5. *Sfrp-1* is expressed in restricted domains in the forebrain and hindbrain. Wholemount in situ hybridization was used to detect *Sfrp-1* transcripts at 7.5 (**A,B**), 8.5 (**C**), 8.75 (**D**), and 9.5 (**E**) dpc. The arrowhead in (E) denotes the junction between the embryonic midbrain and hindbrain. Expression in 10.5 dpc embryos is similar to that seen in 9.5 dpc embryos (not shown). ant, anterior; hp, head process; pe, posterior optic eminence and eye; vhb, ventral hindbrain.

The expression of *mFrzb-1* is seen as early as 7.5 dpc in the primitive streak. Staining is also observed in the prospective cardiac mesoderm at this stage (Figs. 2A,B). By 8.5-9.0 dpc, many somites have been added to the caudal end of the embryo but the neural folds are not completely fused. At this stage, *mFrzb-1* is seen in the presomitic mesoderm from which developing somites are formed (Fig. 2F). In addition, *mFrzb-1* is expressed in the cranial halves of newly formed somites (Fig. 2F). Interestingly, *mFrzb-1* expression in the primitive streak is rather transitory, and by 8.75 dpc, the expression here is no longer detected. In the developing central nervous system (CNS), *mFrzb-1* is found in the dorsal cells of the neural tube in the presumptive midbrain and forebrain at 8.75 dpc (Fig. 2C,E). As the cephalic neural folds begin to fuse anterior to the presumptive midbrain, these dorsal stripes of cells continue to stain strongly for *mFrzb-1* transcripts (Fig. 2E). By 9.0-9.5 dpc, the expression in the dorsal forebrain becomes weaker but still easily detectable.

Expression of mFrzb-1 in Craniofacial Structures

The vertebrate branchial arches, precursors of structures of the craniofacial region, begin to form at approximately 9.0 dpc from mesoderm and neural crest cells migrating underneath the surface ectoderm. By whole-mount in situ hybridization, *mFrzb-1* transcripts are first detected between 8.5 and 8.75 dpc and appear to be restricted to the mesenchymal tissue. By 9.5-9.75 dpc, the expression becomes increasingly apparent at the inferior border of the maxillary division and the superior border of the mandibular division of the first branchial arch (Fig. 3A). The expression of *mFrzb-1* gradually intensifies so that by 10.5 dpc, staining is observed throughout the mesenchyme of the first arch and also extends into the mesenchyme of the hyoid or second branchial arch (Fig. 3B). Intense staining is also observed in the ventral facial mesenchyme adjacent to the maxillary arch (Fig. 3B).

Tooth development commences with a thickening of the oral epithelium to form the developing tooth bud which then involutes into the underlying facial mesenchyme. The thickened epithelium has been shown to secrete several molecules that interact in a reciprocal fashion with the underlying facial mesenchyme (Vainio et al., 1993). At 12.5 dpc, *mFrzb-1* expression is seen in the facial mesenchyme in both the maxillary and mandibular processes. As the epithelium continues to involute, the expression of *mFrzb-1* is restricted to the condensing ectomesenchyme around the presumptive tooth bud (Fig. 3C,D). *mFrzb-1* transcripts are not detected within the epithelial placode.

Boundaries of mFrzb-1 Expression in the Developing Limb

The first discernable area of *mFrzb-1* expression in the developing limbs appears at 9.5 dpc in the ventral mesenchyme of the forelimb bud (Fig. 4A). In the hindlimbs, the expression of *mFrzb-1* transcripts are detected as early as 9.5 dpc. As the limb bud continues to increase in size at 9.75 dpc, the domain of *mFrzb-1* becomes more restricted to the ventral mesenchyme and appears to be excluded from the developing AER (Fig. 3A). By 10.5 dpc, *mFrzb-1* begins to lose its strictly ventral expression pattern in the forelimb but remains ventral in the hindlimb (Figs. 3B,4C). At 11.5 dpc, the ventral expression is no longer detected and the bulk of expression is now seen in the perichondrial regions surrounding the condensing mesenchyme (Fig. 4D). The expression of *mFrzb-1* transcripts in this region appears to preceed overt chondrogenic differentiation, as type II collagen transcripts are only seen in the mesenchymal condensations at this time (Fig. 4F). By 13.5 dpc, *mFrzb-1* expression is found in the proximal and distal ends but not the center of the condensing cartilaginous anlage (Fig. 4G). No *mFrzb-1* transcripts are detected in the surrounding mesenchyme at this time. At this stage, type II collagen transcripts are abundant in the cells of the anlage, indicating that they are undergoing chondrogenic differentiation (Fig. 4H).

Comparison of mFrzb-1 and Wnt Expression Domains

To determine if the localization of *mFrzb-1* occurs at a similar time in a overlapping or complementary pattern as other *Wnt* transcripts, the expression of *mFrzb-1* was compared to that of *Wnt-7a, Wnt-7b, and Wnt-5a*. From 9.5 to 10.5 dpc, *Wnt-7a* expression is consistently restricted to the dorsal ectoderm of the developing limb bud (Fig. 4B; and Parr et al., 1993). This expression pattern is strikingly complementary to the ventrally restricted domain of *mFrzb-1* at these stages (Fig. 4A). At 10.5 dpc, *Wnt-5a* transcripts are expressed in a gradient within the mesenchyme with the highest level in the distal mesenchyme and a lower level proximally. At this stage, *mFrzb-1* expression appears to partially overlap with *Wnt-5a* and begins to switch from its ventral distribution to a region within the perichondrium of the distal mesenchyme (Fig. 4D). By 11.5 dpc, the localization of *mFrzb-1* in the distal mesenchyme clearly shows a partial overlap with that of *Wnt-5a* (Fig. 4D,E).

In the developing CNS, *mFrzb-1* transcripts are consistently observed in the presumptive forebrain just anterior to the forebrain-midbrain junction, and in the midbrain (Fig. 2C). We compared this pattern with that of *Wnt-7b* and found that the transcription of this gene can also be detected in a similar location at the same developmental stage (Fig. 2D).

Sfrp-1 Is Expressed in Specific Regions of the Embryonic Forebrain and Midbrain

Expression of *Sfrp-1* was first detected shortly before formation of the headfolds on day 7 in anterior ectoderm just ahead of the forward-migrating head process (Fig. 5A). At 8.5 dpc, *Sfrp-1* expression was observed in neural ectoderm in the posterior region of the optic

eminences, in the ventral hindbrain, and in the rhombomeres (Fig. 5C,D). An alternate pattern of rhombomeric expression was observed, with Sfrp1 expressed at lower levels in rhombomeres 3 and 5. The relationship of the ectodermal expression on day 7 to expression on later days is not clear. However, the expression seen on day 8.5 in the posterior region of the forming eye and in the ventral hindbrain persists in these structures through the next 48 hours of development (Fig. 5D,E). At 9.5 dpc and later, the anterior limit of *Sfrp-1* expression in the ventral hindbrain corresponds to the junction of the midbrain and hindbrain. No expression of *Sfrp-1* was detected in the developing limbs from 7.0 dpc to 10.0 dpc.

DISCUSSION

Our results indicate that transcripts of *mFrzb-1* and several members of the *Wnt* gene family are detected at similar stages during embryonic development. Within a given tissue, the expression of *mFrzb-1* is either complementary or partially overlapping with *Wnt* gene expression. In the developing limb, *mFrzb-1* expression in the ventral mesenchyme is complementary to that of *Wnt-7a*. *mFrzb-1* expression in the limb and in the CNS shows partial overlap with that of *Wnt-5a* and *Wnt-7b*, respectively. In addition to the limb and CNS expression, transcripts of *mFrzb-1* are also found in the primitive streak, the presomitic mesoderm, cranial region of somites, branchial arches, and in the ectomesenchyme of developing teeth.

Frzb-1 in Limb Development

Wnt-7a plays a pivotal role in specifying the dorsal-ventral axis of the vertebrate limb (Parr and McMahon, 1995). Recent experiments indicate that Frzb-1 physically interacts with Wnt-7a in vitro (K. Lin and F. P. Luyten, unpublished data), and that *mFrzb-1* is expressed at the same stage of limb development as *Wnt-7a* in vivo. However, the expression of *mFrzb-1* in the ventral mesenchyme throughout the early stages of limb morphogenesis is strikingly complementary to that of *Wnt-7a*. This complementary expression pattern of *mFrzb-1* and *Wnt-7a* is similar to that seen with *Frzb-1* and *Wnt-8* in Spemann's organizer (Wang et al., 1997; Leyns et al., 1997). This inverse pattern of *Frzb-1* and *Wnt-7a* expression may enforce a gradient of Wnt-7a activity across the dorsal-ventral axis of the limb, thereby excluding dorsalizing signals from the ventral portion of the limb bud.

As limb morphogenesis progresses, the condensation of mesenchymal cells preceeds chondrogenesis within the limb mesoderm (Hinchliffe and Johnson, 1980). Our data indicate that *mFrzb-1* expression in the limb perichondrium occurs prior to overt chondrogenesis in this area. At later stages, expression is no longer detected in the perichondrium, but is found in the chondroblasts located at the proximal and distal ends of the condensing mesenchyme. This switch in expression pattern may reflect the involvement of Frzb-1 in different developmental processes.

Expression of *Wnt* genes has not been described within the differentiating cartilaginous condensations of the limb to date. In fact, there is evidence to suggest that Wnt-7a from the overlying ectoderm may exert an inhibitory effect on the development of a cartilage phenotype (Rudnicki and Brown, 1997). Ectopic expression of *Wnt-1* and *Wnt-7a* genes in the limb bud and facial primordium causes inhibition of chondrogenesis and proliferation of undifferentiated mesenchyme in these regions (Rudnicki and Brown, 1997). It is therefore conceivable that Frzb-1 may bind to Wnt-7a and exclude Wnt activity from the condensing cartilaginous cores.

Within the limb mesenchyme, a partial overlap exists between the expression domain of *mFrzb-1* and that of *Wnt-5a*. Frzb-1 also binds directly to Wnt-5a but fails to suppress the phenotypic changes induced by Wnt-5a in Xenopus embryos (Lin et al., 1997). These results imply that interactions between Frzb-1 and Wnt family members do not necessarily result in inhibition of Wnt activity. Alternatively, it has been suggested that Frzb-1 may bind certain Wnt proteins to serve a chaperone function (Leyns et al., 1997). The present colocalization of *mFrzb-1* and *Wnt-5a*, together with the observation that Frzb-1 binds to Wnt-5a and yet fails to inhibit its activity, does not contradict this hypothesis of a chaperone function in some tissues.

Of considerable interest is the observation that *Frzb-1* expression not only persists in the epiphyseal cartilage postnatally, but this expression of *Frzb-1* in cartilage is more abundant than in any other postnatal tissue studied. This observation is intriguing, given that expression of *Wnt* genes has not been reported in this region to date.

Frzb-1 in Craniofacial and CNS Development

There is evidence to suggest that epithelial-mesenchymal interactions play a major role in the formation of the vertebrate tooth. The early patterning for tooth development is initiated by signals coming from the oral epithelium (Lumsden, 1988). The oral epithelium first thickens locally and then invaginates into the underlying mesenchyme to form a tooth bud. The ectomesenchyme then undergoes a process of condensation during which time the upregulation of several signaling molecules and transcription factors are observed (Wilkinson et al., 1989; Pelton et al., 1990; Karavanova et al., 1992; Jowett et al., 1993). There is evidence that members of the bone morphogenetic protein (BMP) family mediate these epithelial-mesenchymal interactions during tooth development (Vainio et al., 1993). The observation that *mFrzb-1* is present in the condensing ectomesenchyme when these interactions occur points to a role of this gene in early tooth development. Given the affinity of Frzb-1 for Wnt proteins, it is possible that the mechanisms underlying tooth development also involve the modulation of Wnt

activity by Frzb-1. Previous studies have localized *Wnt-5a* and *Wnt-7a* transcripts to the developing branchial arches (Dealy et al., 1993). However, our localization studies did not reveal expression of either *Wnt-5a* or *Wnt-7a* in the ectomesenchyme of the developing tooth at 12.5 dpc. Our data do not exclude the possible involvement of other Wnt family members in tooth development.

As the embryonic brain develops, it partitions mainly into three regions: the forebrain, the midbrain, and the hindbrain. Subsequently, these regions subdivide to form distinct and more specialized structures of the embryonic and adult brain. The restricted expression of *mFrzb-1* appears at early stages of forebrain and midbrain development, suggesting a role in early development of these areas of the CNS.

Unique Expression Pattern of frzb Family Members

Recently a family of molecules has been described which shares the CRD of the Wnt receptor Frizzled, but lacks the putative transmembrane domain. To date, this family includes the proteins Frzb-1 (Hoang et al., 1996), SDF5 (Shirozu et al., 1996) Sfrp-1 and Sfrp-4 (Rattner et al, 1997), Crescent (Pfeffer et al., 1997), and SARP3 (Melkonyan et al., 1997). In order to begin to define physiological roles for these proteins, it is necessary to determine whether they are coexpressed in similar tissues, or whether each of these proteins has a unique chronological or tissue specific distribution pattern.

Our data indicate that the spatiotemporal pattern of gene expression varies considerably between the two Frzb-related molecules examined in this study. The expression of *Frzb-1* is found primarily in the branchial arches, forebrain, and developing limbs. In contrast, the distribution of *Sfrp-1* is primarily in the forebrain, midbrain, and posterior eye structures, but is undetectable in the limbs. *Crescent* expression has been localized to the primitive streak and cells which give rise to the presumptive foregut. Taken together, these results indicate that this emerging family of Frzb-related molecules may be performing similar roles in different tissues at different times.

On the basis of these results, the interaction of Frzb-1 and Wnt family members is likely to be complex and dynamic, depending on the tissue or the developmental stage in which these molecules exert their actions. This complexity is compounded by the existence of multiple secreted Frizzled-like molecules (Rattner et al., 1997) and a large family of Wnt proteins. Future experiments will address both the nature and the specificity of these important interactions during development.

EXPERIMENTAL PROCEDURES

Cloning of Mouse Frzb-1

A murine 129/sv genomic DNA library in λ Fix II (Stratagene, La Jolla, CA) was screened using 604 basepairs (bp) of a α-^{32}P-dCTP-labelled bovine *Frzb-1* cDNA probe (nt 344-948 in Hoang et al., 1996). One genomic clone (mFrzb 3.1) containing a 20-kb insert was characterized further and was found to contain exon 1 of murine *Frzb-1*. A 600-bp cDNA fragment containing exon 1 was amplified by polymerase chain reaction (PCR) using the mouse genomic clone as a template. The PCR product was subcloned into the Srf1 site of PCR-Script (Stratagene) and the resulting construct was named *mFrzb-1*. A SmaI-restricted fragment of *mFrzb-1* was used as a probe for Northern analysis.

Northern Blot Analysis

Tissues were rapidly frozen in dry ice, homogenized in 6 M guanidine HCl, followed by cesium chloride gradient centrifugation and sequential lithium chloride/ethanol precipitation. Five to 20 micrograms of total cellular RNA per lane were separated by 1% agarose gel electrophoresis. Ethidium bromide staining of 28S and 18S ribosomal RNA bands and hybridization with α-^{32}P-dCTP-radiolabelled glyceraldehyde-6-phosphate dehydrogenase (GAPDH) cDNA were performed to assess the equivalence of loading of RNA in each lane. The separated RNA was then transferred to Nytran(Plus membrane (Schleicher & Schuell, Keene, NH) by capillary action and crosslinked by UV light. A SmaI-restricted fragment of *mFrzb-1* was radiolabelled with α-^{32}P-dCTP using the random priming technique (Gibco BRL, Gaithersburg, MD). Membranes were hybridized and washed according to established methods (Church and Gilbert, 1984). All autoradiography was performed using intensifying screens at −70°C

In Situ Hybridization

mFrzb-1 was linearized with NotI and transcribed with T7 RNA polymerase for the antisense probe or linearized with EcoRI and transcribed with T3 RNA polymerase for the sense control probe. Digoxigenin-UTP-labelled antisense and sense probes were synthesized using the Genius™ 4 System (Boehringer Mannheim, Indianapolis, IN). Other probes used in this study were as follows: full-length *Sfrp-1* (Rattner et al., 1997; a generous gift from Dr. Jeremy Nathans, Johns Hopkins Univeristy); 405 bp of 3′-UTR of the murine type II collagen cDNA (a generous gift from Dr. Suneel Apte, Cleveland Clinic Foundation, Cleveland, OH); a full-length murine *Wnt-5a* cDNA (Gavin et al., 1990); a full-length murine *Wnt-7a* cDNA (Gavin et al., 1990); a murine *Wnt-7b* cDNA (306 bp of 3′-UTR, Parr et al., 1993). All Wnt cDNAs were generous gifts from Dr. Andrew McMahon, Harvard University.

Wholemount in situ hybridization using CD1 mouse embryos were performed as described by Henrique et al. (1995) with slight modifications. After fixation in 4% paraformaldehyde-phosphate-buffered saline (PBS), embryos were bleached with methanol:30% H_2O_2 (5:1). Embryos were permeabilized with proteinase K (10 μg/ml, Sigma, St. Louis, MO) for 6-12 min, followed by a brief rinse in PBS-0.1% Tween 20 (PBT), and immediate fixation in 4% paraformaldehyde plus 0.1% gluteral-

dehyde in PBT. Embryos were then washed once with hybridization buffer (50% formamide, 1.3× SSC, pH 5.0, 5 mM EDTA, 50 µg/ml yeast tRNA, 0.2% Tween 20, 0.5% 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonic acid (CHAPS), 100 µg/ml Heparin) and incubated at 65°C for 1 hr in hybridization buffer. Digoxigenin-labelled riboprobes were then added at 1-1.5 µg/ml and embryos incubated overnight at 65°C. Following hybridization, embryos were washed twice at 65°C for 10 min with hybridization buffer, twice for 30 min with wash solution I (50% formamide, 1X SSC, 0.1% Tween 20), and three times for 10 min with MABT (100 mM maleic acid, 150 mM NaCl, 0.1% Tween 20, pH 7.5). Blocking of nonspecific antibody binding was performed in MABT-2% Boehringer blocking reagent (BBR) for 1-2 hr followed by incubation in MBS (MABT, 1% BBR, 20% goat serum heat-treated for 30 min at 56°C) for 1 hr. Embryos were then incubated overnight at 4°C in MBS with anti-digoxigenin alkaline phosphatase conjugate (Boehringer Mannheim). After antibody incubation, embryos were washed three times with MABT for 1 hr and twice with NTMT (100 mM NaCl, 100 mM Tris-HCL, 50 mM $MgCl_2$, 1% Tween 20) for 10 min. Color reactions were then developed in 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). After reactions were complete, embryos were washed in PBT overnight and cleared with 50% glycerol in PBT followed by 80% glycerol in PBT. Embryos were photographed in 80% glycerol in PBT.

For in situ hybridization of tissue sections, embryos were fixed in 4% paraformaldehyde in PBS, embedded in optimal cutting temperature (OCT), and sectioned at 6-15 µm. After digestion with proteinase K, sections were washed quickly in PBS and then immediately fixed in 4% paraformaldehyde-PBS. Digoxigenin-labelled probes were dissolved in hybridization buffer (50% formamide, 10 mM Tris-HCl, pH 7.6, 200 mg/ml yeast tRNA, 1X Denhardt's, 10% dextran sulfate, 600 mM NaCl, 0.25% SDS, 1 mM EDTA, pH 8.0). The hybridization mix was briefly boiled and applied directly onto sections. After hybridization for 12-16 hr at 53°C, the following washing procedure was used: 5X SSC at 56°C for 1 min; 2X SSC at 56°C for 30 min; TNE (10 mM Tris, pH 7.5, 0.5 M NaCl, 1 mM EDTA) at 37°C for 10 min; TNE with 10 µg/ml RNAse A at 37°C for 30 min; TNE at 37°C for 10 min; 2X SSC at 53°C for 20 min; 0.2X SSC at 53°C, twice for 20 min. Blocking of nonspecific antibody binding was performed for 1-2 hr with 2% BBR in DIG1 buffer (100 mM Tris-HCL, pH 7.5, 150 mM NaCl). Sections were counterstained with methyl green and mounted with Permount. Brightfield photographs were taken with a Nikon Optiphot-2 microscope and Kodak Ektachrome 160T slide film.

ACKNOWLEDGMENTS

We thank B. Mita, X. Zhou, J. Welter, and Yee Hsee Hsieh for expert technical assistance. We thank A. McMahon for the *Wnt-5a*, *7a*, and *7b* clones, S. Apte for the mouse type II collagen clone, and J. Nathans for the *Sfrp-1* clone. B.H.H is supported by the Allen Fellowship from the Department of Orthopaedics at Case Western Reserve University, Cleveland, Ohio. Funding was provided by University Hospitals of Cleveland and the Rainbow Babies and Childrens Hospital.

REFERENCES

Bhanot P, Brink M, Samos CH, Hsieh JC, Wang Y, Macke JP, Andrew D, Nathans J, Nusse R. A new member of the frizzled family from Drosophila functions as aWingless receptor. Nature 1996;382:225–230.

Church GM, Gilbert W. Genomic sequencing. Proc. Natl. Acad. Sci. USA 1984;81:1991–1995.

Cohn MJ, Tickle C. Limbs: a model for pattern formation within the vertebrate body plan. Trends Genet. 1996;12:253.

Dealy NC, Roth A, Ferrari D, Brown AMC, Kosher RA. Wnt-5a and Wnt-7a are expressed in the developing chick limb bud in a manner suggesting roles in pattern formation along the proximodistal and dorsoventral axes. Mech. Dev. 1993;43:175–186.

Finch PW, He X, Kelly MJ, Uren A, Schaudies RP, Popescu NC, Rudikoff S, Aaronson SA, Varmus HA, Rubin JS. Purification and molecular cloning of a secreted, Frizzled-related antagonist of Wnt action. Proc. Natl. Acad. Sci. USA 1997;94:6770–6775.

Gavin BJ, McMahon JA, McMahon AP. Expression of multiple novel Wnt-1/Int-1-related genes during fetal and adult mouse development. Genes Dev. 1990;4:2319–2332.

Henrique D, Adam J, Myat A, Chitnis A, Lewis J, Ish-Horowicz D. Expression of a Delta homologue in prospective neurons in the chick. Nature 1995;375:787–790.

He X, Saint-Jeannet JP, Wang Y, Nathans J, Dawid I, Varmus H. A Member of the Frizzled Protein Family Mediating Axis Induction by Wnt-5A. Science 1997;275:1652–1654.

Hoang B, Moos M, Jr, Vukicevic S, Luyten FP. Primary Structure and Tissue Distribution of FRZB, a Novel Protein Related to Drosophila Frizzled Suggest a Role in Skeletal Morphogenesis. J. Biol. Chem. 1996;271:26131–26137.

Hinchliffe JR, Johnson DR. The Development of the Vertebrate Limb. Oxford University Press, New York, 1980.

Jowett AK, Vainio S, Ferguson MWJ, Sharpe PT, Thesleff I. Epithelial-mesenchymal interactions are required for msx 1 and msx 2 gene expression in the developing murine molar tooth. Development 1993;117:461–470.

Karavanova I, Vainio S, Thesleff I. Transient and recurrent expression of the Erg-1 gene in epithelial and mesenchymal cells during tooth morphogenesis suggests involvement in tissue interactions and determination of cell fate. Mech. Dev. 1992;39:41–50.

Leyns L, Bouwmeester T, Kim SH, Piccolo S, De Robertis EM. Frzb-1 Is a Secreted Antagonist of Wnt Signaling Expressed in the Spemann Organizer. Cell 1997;88:747–756.

Lin K, Wang S, Julius MA, Kitajewski J, Moos M, Jr, Luyten FP. The cysteine-rich frizzled domain of Frzb-1 is required and sufficient for modulation of Wnt signaling. Proc. Natl. Acad. Sci. USA 1997;94: 11196–11200.

Lumsden AGS. Spatial organization of the epithelium and the role of neural crest cells in the initiation of the mammalian tooth germ. Development 1988;103:155–169.

Mayr T, Deutsch U, Kuhl M, Drexler HCA, Friedrich L, Deutzmann R, Wedlich D, Risau W. Fritz: a secreted frizzled-related protein that inhibits Wnt activity. Mech. Dev. 1997;63:109–125.

McMahon AP, Bradley A. The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. Cell 1990;62: 1073–1085.

Melkonyan HS, Chang WC, Shapiro JP, Mahadevappa M, Fitzpatrick PA, Kiefer MC, Tomei LD, Umansky SR. SARPs: A family of secreted apoptosis-related proteins. Proc. Natl. Acad. Sci. USA 1997;94:13636–13641.

Munsterberg AE, Kitajewski J, Bumcrot DA, McMahon AP, Lassar AB. Combinatorial signaling by Sonic hedgehog and Wnt family mem-

bers induces myogenic bHLH gene expression in the somite. Genes Dev. 1995;9:2911–2922.
Niswander L, Tickle C, Vogel A, Booth I, Martin GR. FGF-4 Replaces the Apical Ectodermal Ridge and Directs Outgrowth and Patterning of the Limb. Cell 1993;75:579–587.
Nusse R, Varmus HE. Wnt genes. Cell 1992;69:1073–1087.
Parr BA, McMahon AP. Dorsalizing signal Wnt-7a required for normal polarity of D-V and A-P axes of mouse limb. Nature 1995;374:350–353.
Parr BA, Shea MJ, Vassileva G, McMahon AP. Mouse Wnt genes exhibit discrete domains of expression in the early embryonic CNS and limb buds. Development 1993;119:247–261.
Pelton RW, Dickinson ME, Moses HL, Hogan BLM. In situ hybridization analysis of TGFβ3 RNA expression during mouse development: comparative studies with TGFβ1 and β2. Development 1990;110: 609–620.
Perrimon N. Serpentine Proteins Slither into the Wingless and Hedgehog Fields. Cell 1996;86:513–516.
Pfeffer PL, De Robertis EM, Izpisua-Belmonte JC. Crescent, a novel chick gene encoding a Frizzled-like cysteine-rich domain, is expressed in anterior regions during early embryogenesis. Int. J. Dev. Biol. 1997;41:449–458.
Rattner A, Hsieh JC, Smallwood PM, Gilbert DJ, Copeland NG, Jenkins NA, Nathans J. A family of secreted proteins contains homology to the cysteine-rich domain of frizzled receptors. Proc. Natl. Acad. Sci. USA 1997;94:2859–2863.
Riddle RD, Johnson RL, Laufer E, Tabin C. Sonic hedgehog mediates the polarizing activity of the ZPA. Cell 1993;75:1401–1416.
Riddle RD, Ensini M, Nelson C, Tsuchida T, Jessell TM, Tabin C. Induction of the LIM Homeobox Gene Lmx1 by WNT7a Establishes Dorsoventral Pattern in the Vertebrate Limb. Cell 1995;83:631–640.
Rudnicki JA, Brown AMC. Inhibition of Chondrogenesis by Wnt Gene Expression in Vivo and in Vitro. Dev. Biol. 1997;185:104–118.
Shirozu M, Tada H, Tashiro K, Nakamura T, Lopez ND, Nazarea M, Hamada T, Sato T, Nakano T, Honjo T. Characterization of novel secreted and membrane proteins isolated by the signal sequence trap method. Genomics 1996;37:273–280.
Thomas KR, Capecchi MR. Targeted disruption of the murine int-1 proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. Nature 1990;346:847–850.
Vanio S, Karavanova I, Jowett A, Thesleff I. Identification of BMP-4 as a Signal Mediating Secondary Induction between Epithelial and Mesenchymal Tissues during Early Tooth Development. Cell 1993; 75:45–58.
Vinson CR, Adler PN. Directional non-cell autonomy and the transmission of polarity information by the frizzled gene of Drosophila. Nature 1987;329:549–551.
Vinson CR, Conover S, Adler PN. A Drosophila tissue polarity locus encodes a protein containing seven potential transmembrane domains. Nature 1989;338:263–264.
Wang S, Krinks M, Lin K, Luyten FP, Moos M, Jr. Frzb, a Secreted Protein Expressed in the Spemann Organizer, Binds and Inhibits Wnt-8. Cell 1997;88:757–766.
Wang-Snyder J, Miller JR, Brown JD, Lai C-J, Moon RT. A frizzled homolog functions in a vertebrate Wnt signaling pathway. Current Biol 1996;6:1302–1308.
Wilkinson DG, Bhatt S, McMahon AP. Expression pattern of the FGF-related proto-oncogene int-2 suggests multiple roles in fetal development. Development 1989;105:131–136.
Zakany J, Duboule D. Correlation of expression of Wnt-1 in developing limbs with abnormalities in growth and skeletal patterning. Nature 1993;362:546–549.